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ANNUAL REPORT

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PRINCIPAL INVESTIGATOR: Hans Pepper, M.D.

INSTITUTION: The Mount Sinai Hospital, New York

SUBJECT: Histopathology of Germfree Animals

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A B S T R A C T

1. Institution: The Mount Sinai Hospital, New York
2. Title: Histopathology of Germfree Animals
3. Principal Investigators: Hans Popper, M.D.,
Heinz Bauer, M.D., Richard E. Horowitz, M.D.
and Fiorenzo Paronetto, M.D.
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Quantitative histologic, immunocytochemical, autoradiographic and serologic studies on germfree and conventional animals subjected to various stresses were performed to assay and separate function and reaction of immunologically competent cell elements and phagocytic elements of the reticuloendothelial system. Lymph nodes and spleen of germfree animals exhibit a normal complement of macrophages, but reduced number of plasma cells and precursors. In both the germfree and conventional animal, the lymph nodes draining the intestine are more active than peripheral nodes. However, the phagocytic and immunologically competent cell systems are underdeveloped in the wall of the germfree intestine. Local administration of bacterial antigen elicits equal serologic and structural response of the immunologically competent cell in germfree and conventional animals. The known plasma cell reaction and rise in serum gamma globulin following sublethal radiation is of equal magnitude in germfree and conventional animals, also indicating a normal responsiveness of the underdeveloped immunologically competent cell system. It also suggests that this response does not result from bacterial action but cell breakdown on either a metabolic or autoimmune basis. Thus, the small number and virginal state of immunologically competent cells in the germfree animal does not interfere with normal reaction to stress.

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I. PURPOSE:

This study was designed to describe the microscopic, histochemical and immunocytochemical structure of the reticuloendothelial system of normal germfree, monocontaminated (with *Escherichia coli*) and conventional mice, with particular emphasis on the alterations in morphology and function resulting from various experimental stresses such as whole body irradiation, antigenic challenge, endotoxin shock, wound healing and liver injury.

II. COMPLETED STUDIES:

A. RETICULOENDOTHELIAL SYSTEM OF UNSTRESSED GERMFREE MICE.

1. Light Microscopy: Seventy six mice of two strains were studied to establish the appearance of the spleens and lymph nodes draining the gastrointestinal tract versus nodes draining other parts of the body. The relative distribution of the various cells was quantitated by conventional microscopy. In germfree mice the lymph nodes draining the gastrointestinal tract revealed a distribution of macrophages similar to conventional animals, presumably the result of irritation by material not derived from viable bacteria. In contrast, plasma cells and precursors are much sparser in germfree lymph nodes. In nodes not draining the gastrointestinal tract, conventional animals showed fewer plasma cells and germfree nodes virtually none. Lymph nodes and spleens of the germfree mice contained more lymphatic tissue but fewer reaction centers than those of the conventional animals. This specific variation of activity of mesenchymal elements differentially affecting the phagocytic and immunologically competent cells in germfree animals serves as a baseline to study responses to injury.

2. Immunocytochemistry: The distribution of gamma globulin in the gastrointestinal tract lymph nodes, the systemic lymph nodes and spleen was studied in random germfree and conventional mice by routine immunocytochemical techniques, and was correlated with quantitative morphology under the light microscope. Plasma cells showing specific fluorescence when stained with rabbit anti-mouse gamma

globulin were numerous in the gastrointestinal tract lymph nodes of conventional mice but virtually absent in the germfree. Such gamma globulin containing cells were not found in the spleens of either conventional or germfree mice. In lymph nodes anatomically unrelated to the gastrointestinal tract, there were rare plasma-cytoid cells showing fluorescence in the conventional but virtually none in the germfree. Although the number of plasma cells was always markedly lower in the germfree mice, the number of plasma cells with Russel bodies was greater. The scarcity of demonstrable gamma globulin in the lymphatic tissue of germfree mice accompanies the known hypo-gamma globulinemia which these animals demonstrate,, presumably due to lack of antigenic stimulation by the bacterial microflora. The absence of viable bacteria does not affect the phagocytic function of lymphatic tissue.

3. Autoradiography: Sixty six unstressed germfree and conventional Swiss Webster mice were given 40 microcuries of tritiated thymidine intraperitoneally. Animals were sacrificed 1, 10, 24 and 48 hours after injections, and lymph nodes and spleens were studied. Most cells labelled with tritium were either hematopoietic or precursor cells of the plasma cell series. Labelled hematopoietic cells, when present, were located exclusively in medullary cords of lymph nodes and in the red pulp of the spleen, while labelled immunocytes were found in the lymph node cortex and in splenic follicles; in the lymph nodes they were, in addition, noted at the corticomedullary junction and in the contiguous portions of the medullary cords. In conventional mice labelled immunocytes

were more numerous in the lymph nodes draining the gastrointestinal tract and less frequent in nodes draining the extremities. Germfree lymph nodes, irrespective of drainage area, showed few such labelled cells. The cells which were found to have incorporated the label, one hour following injections of tritiated thymidine can be considered to identify the most actively proliferating cell population. These cells were identical in incidence, location and type with the immunocytes observed by routine microscopy and immunocytochemistry. Thus, short term autoradiography gives a precise indication of the immunologic activity of lymphatic tissue.

4. Summary: The number, distribution, histologic and histochemical characteristics of macrophages in lymph nodes and spleens of germfree and conventional mice did not differ; in lymph nodes the macrophages were found to be related only to the area of drainage, being more prominent in nodes related to the oropharynx and intestine than in those related to the extremities.

Reaction centers and immunologically competent cells, however, whether defined as Marschalko plasma cells on conventional microscopy or as gamma globulin-containing cells by immunocytochemistry, were rare in germfree lymph nodes and spleens and abundant in comparable tissues of conventional mice.

This indicates that the presence of a microbial flora affects the immunologic but not the phagocytic function of lymph nodes and spleens.

B. GASTROINTESTINAL TRACT OF UNSTRESSED GERMFREE MICE.

1. This study was performed in collaboration with the Department of Experimental Pathology, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D. C.

2. Sixty six germfree and conventional Swiss Webster mice were given 40 microcuries of tritiated thymidine intraperitoneally and were sacrificed 1, 10, 24 and 48 hours after injection. The terminal ileum was studied histologically and radioautographically.

3. The study revealed that the intestine of the conventional animal possesses a bulkier and more cellular lamina propria than the germfree and that the mitotic rate in the intestinal crypts of conventional mice is significantly greater than in germfree, indicating that the intestinal flora, even in the unstressed animal accelerates mucosal cell replacement. The intestinal flora also affects the kinetics of mucosal cell migration and replacement. Autoradiographic studies showed that the turnover of ileal epithelium of the germfree mouse is approximately half of the conventional animal. With regard to the lamina propria, the conventional state appears to be one of "physiologic inflammation". In germfree mice the lamina propria contains virtually no reticuloendothelial cells and the lymphatic tissue is devoid of plasma cells and their precursors. The lymphatic tissue of the germfree intestine also contained very few labelled cells indicating the virtual absence of immunologic phenomena.

C. RESPONSE OF GERMFREE LYMPHATIC SYSTEM TO ANTIGENIC CHALLENGE

1. Introduction: This study was designed to assay the response of a previously inexperienced lymphatic system, as in germ-free animals, to bacterial antigens. This was done by determining (1) the distribution and fate of injected bacteria, (2) the appearance and distribution of immunocytes (3) the appearance of reaction centers and (4) the development of circulating antibody.

2. Methods: Seventy seven germfree and 90 conventional ND2 mice were sacrificed 2, 6, 12, and 24 hours, and 2, 4, 7, and 14 days after the injection of 0.05 cc. of *E. coli* OB antigen into the left paw. One hour before sacrifice, the animals in the 24 hour and the 4, 7, and 14 day group received an intravenous injection of 40 mc of tritiated thymidine. Serum was collected for antibody titer determination. Bilateral axillary and brachial lymph nodes and spleens were removed. Alternating pairs of left and right axillary or brachial nodes and half the spleen were studied immunocytochemically for antigen and gamma globulin. The remainder of the spleen, and the other pair of axillary or brachial lymph nodes were stained with hematoxylin-eosin, giemsa, periodic acid-Schiff and methyl green pyronine; radioautographs were also prepared.

3. Results: All nodes on the injected side enlarged after 6 hours and doubled their weight by 4 days. Particulate antigen appeared in sinus macrophages of all nodes on the injected side after 2 hours but appeared to disintegrate more rapidly in conventional macrophages. Plasma cell precursors and cells labelled with

tritiated thymidine appeared in the same areas and in similar numbers in both germfree and conventional nodes, reaching their peak at 7 days. Reaction center, mature plasma cells and gamma globulin containing cells increased after 4 days and also reached a maximum at 7 days in both groups. Circulating antibody was present in all mice with higher titers in the conventionals.

4. Summary: Germfree and conventional lymph nodes take up bacterial antigen rapidly with a similar sequence of morphologic and serologic events, with the possible exception of slower disintegration of antigen in germfree macrophages. Under the conditions of this experiment, the underdeveloped immunologic system of the germfree animal responds to challenge like the immunologically experienced conventional.

III. STUDIES IN PROGRESS

A. RESPONSE OF LYMPHATIC SYSTEM TO WHOLE BODY IRRADIATION.

Plasma cell proliferation in lymphatic tissue and serum gamma globulin elevation following whole body irradiation of conventional animals is considered an antibody response to invading microorganism. To test this hypothesis, 120 germfree and conventional mice received 550 roentgens whole body x-radiation and were sacrificed at intervals. Blood was taken for electrophoresis; lymph nodes and spleens were weighed and studied by immunocytochemistry, autoradiography and quantitative histology.

Disappearance of lymphocytes in all lymph nodes and spleens occurred two days after irradiation; near normal numbers of lymphocytes were present at 15 days. Weight loss of lymph nodes and spleens two days after irradiation with return toward normal at 15 days corresponded to disappearance and reappearance of lymphocytes. Plasma cells and gamma globulin containing cells proliferated in germfree and conventional mice, and at 10 days such cells were the major constituents of the medullary cords.

The presence of living bacteria, therefore, does not explain the morphologic response of the lymphatic system following radiation, as assumed until now; rather, an effect of cell breakdown products, possibly from lymphocytes, upon immunologically competent cells is postulated. Further investigation, including cell derivation studies, may show these phenomena to be either autoimmune or metabolic reactions, of which the initially underdeveloped lymphatic system of the germfree animal is equally capable. As in previous studies at this dose, mortality was confined to conventional mice; all germfree animals survived. The lymphatic response, as determined in this study, does not explain the radioresistance of the germfree animals. The definition of the precise role of the immunologic phenomena in the response to irradiation are under study.

B. RESPONSE OF LYMPHATIC SYSTEM TO LIVER INJURY.

Increase in the size of the lymphoid system and serum gamma globulin elevation are common findings in chronic liver disease in man and experimental animals. The etiology of the gamma globulin

elevation and concomitant pulp cell hyperplasia in spleen and lymph nodes in natural or experimental liver disease is not clear; it may (1) represent antibody against liver and thus implicate auto-immune mechanisms in the chronicity of liver disease; (2) represent an anamnestic response to normally occurring microbial antigens with liver injury providing an adjuvant-like effect; (3) reflect a change in the bacterial flora secondary to liver disease with predominance of more antigenic organisms or (4) represent a decreased catabolism or a non-specific increase in synthesis of gamma globulin.

Since such an experiment had never been performed in isolator maintained animals, and in order to determine the morality and the most appropriate times for sacrifice, a group of 63 germfree and conventional ICR strain mice were given 0.03 ml carbon tetrachloride subcutaneously, twice weekly, beginning at age of 10-12 weeks. Mice were sacrificed 2, 3, 4, 8, 12 and 14 weeks later and liver spleen and lymph nodes were weighed and prepared for routine microscopy, histochemistry and immunocytochemistry.

A striking mortality of approximately 50% was seen during the first month, in germfree and in isolator maintained conventional mice. Cirrhosis was seen in germfree males and females and in conventional males after 12 weeks of carbon tetrachloride administration. Conventional female mice did not show cirrhosis at 14 weeks. The hepatic lymph nodes of germfree and isolator reared conventional mice showed a moderate plasmacytosis beginning at 8 weeks. Serum gamma globulin did not change significantly in conventional mice; however, there was a slight rise in the germfree beginning 4 weeks after carbon tetrachloride.

In a similar experiment on conventional mice kept in open animal rooms (at the Mount Sinai Hospital) there was a definite cirrhosis at 3 weeks and a marked plasmacytosis of the lymph nodes and significant elevation of serum gamma globulin beginning at 4 weeks.

Since the conventional mice maintained in isolators showed no elevation in serum gamma globulin while mice maintained in open animal rooms do show a rise, further groups of open and isolator maintained conventional mice are being studied. The slight rise in gamma globulin shown by germfree mice suggests that bacteria do not play a significant role, however, larger number of animals, sacrificed 1, 2 and 3 months after injection must be studied to extend and confirm these preliminary results.

IV. STUDIES IN PREPARATION

A. THE IMMUNOLOGY OF WOUND HEALING.

The rate of wound healing and the tensile strength of wounds is markedly reduced in germfree animals. The cause of this healing deficit is not clear. It may be due to the absence of irritating and stimulating microorganisms or it may be due to a basic metabolic difference in the response of the lymphoid system to injury.

A small number of germfree mice have recently been found with self-inflicted chronic peri-auricular wounds. Extensive bacteriologic and histologic examination revealed no evidence of bacterial contamination. Lymph nodes draining the site of injury were larger than their opposite counterpart in the same anatomic location,

and contained many very active reaction centers and sheets of plasma cells. The axillary and popliteal lymph nodes of these animals showed more plasma cells than usual in germfree animals but their number did not approach that of the cervical nodes which came from the drainage area of the lesions. The spleens and mesenteric nodes were not remarkable and showed the usual structure of germfree lymphatic tissue.

To ascertain the role of the lymphatic tissue in the local response to injury, this study will utilize standard incisions, application of necrotizing chemicals such as croton oil and subcutaneous deposition of irritants such as turpentine. The lesions will be placed in the drainage area of the axillary lymph nodes which will be studied histologically, histochemically and immunocytochemically. Comparison of germfree and conventional animals will further define the role of the bacterial flora and in the immunologic phenomena which are involved.

B. RESPONSE OF LYMPHATIC SYSTEM TO ADRENOCORTICAL STEROIDS

Adrenocortical hormones influence the entire spectrum of responses to injury. Resistance to microorganisms is often decreased and intercurrent infections complicate experiments which try to define broader aspects of corticoid effect.

Studies of lymphatic tissue in steroid-treated conventional animals are often difficult to evaluate because hormone effect as such can not be distinguished from coinciding response to microbial invasion. Germfree animals exclude this variable and permit more precise observations of cellular and functional morphology.

Groups of germfree and conventional mice will receive graded doses of cortisone and matched groups will be injected with saline. The animals will be studied histologically, biochemically, and immunologically and, in addition, body weight and survival times will also be recorded. It is expected that germfree mice will lose less weight, survive longer, and show fewer changes in all parameters studied.

C. RESPONSE OF LYMPHATIC TISSUE TO AGING, ENDOTOXIN AND HOMOTRANSPLANTATION.

The unstimulated lymphatic tissue of germfree animals serves as a pure and well controlled subject for study of aging processes, particularly as related to the phenomenon of immunological tolerance, and for studies of the response to external stresses such as endotoxin and tissue transplantation. Such studies, as described in previous reports, are currently under consideration.

One additional study, currently in the planning stage, is the evaluation of the role of the microbial flora and endotoxin in the etiology of cirrhosis. Fine and Rutenberg and recently Broitman (Fed.Proc. 22:511, 1963) have suggested that either pathogenic microorganisms or oral endotoxin are essential, together with dietary deficiency in the etiology of cirrhosis. Thus germfree and conventional rats will be given either endotoxin or carbon tetrachloride or both in an attempt to evaluate the role of the hepatotoxin, the microbial flora and endotoxin in the etiology of chronic liver disease.

V. PUBLICATIONS AND PRESENTATIONS

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Matkins, K.C., Levenson, S.M. and Popper, H.: Immunologic
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without stress. (To be presented at the Multiple Disci-
pline Research Forum at the 1963 Annual Meeting of the
American Medical Association, 20 June 1963, Atlantic City,
N.J.)

VI SUMMARY

To date these studies with germfree animals have provided information of major importance to military medicine, particularly in the broad area of reaction to stress. Thus it has been found that the lymphatic tissue of animals reared in the total absence of a microbial flora is deficient in immunologically competent cells and in humoral factors such as antibody and gamma globulin which are related to the defense mechanism. However, when adequately stimulated these animals, in spite of their prior inexperience, show morphologic and humoral responses which are qualitatively indistinguishable from those of experienced conventional animals.

This uptake of bacterial antigen and the antibody response to such antigen is essentially the same in germfree and conventional animals. The response of the lymphatic system to toxic liver injury appears to be similar in germfree and conventional animals. The local response to trauma although quantitatively different is qualitatively the same in germfree and conventional animals and, finally, the response of the lymphatic tissue to irradiation is identical in germfree and conventional animals.

These studies suggest therefore, that the role which lymphatic tissue plays in the reaction to various forms of stress and trauma is relatively independent of prior experience with the normal microbial flora.

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